Applicability of cord blood-derived unrestricted somatic stem cells in tissue engineering concepts

Ö. Degistirici*·†, M. Jäger‡ and A. Knipper*

*Kourion Therapeutics GmbH, Langenfeld, Germany, †Center of Advanced European Studies and Research, Bonn, Germany, and ‡Department of Orthopaedics, Heinrich-Heine University Hospital Düsseldorf, Düsseldorf, Germany

Received 10 August 2007; revision accepted 2 November 2007

Abstract. *Objectives:* Cell-based tissue engineering concepts are becoming an important therapeutic alternative in the treatment of traumatic or chronic skeletal diseases. Here, we have evaluated cord blood-derived unrestricted somatic stem cells (USSCs) for use in bone and cartilage repair strategies. *Methods and results:* This type of somatic stem cell can be generated from cord blood with a current rate of 29% and we have documented excellent proliferation potential to high passage numbers. The cells have an initial population doubling time of 39 h, which slightly decreased with increasing passage number, but cells maintained their proliferation abilities up to passage 23. Cells clearly differentiated towards chondrogenic, adipogenic and osteogenic lineage as shown by reverse transcription-polymerase chain reaction as well as by histological, biochemical and immunohistochemical stains. Differentiation potential of USSCs was observed at passage 6, passage 15 and passage 21. In addition, USSCs showed increased secretion of vascular endothelial growth factor (VEGF) during osteogenic differentiation, as well as expression of key markers of angiogenesis such as vascular endothelial growth factor receptor-2 and platelet/endothelial cell adhesion molecule. *Conclusions:* USSCs when transplanted into a bone defect might support the repair process not only by pure remineralization but also by installation of angiogenic environment.

INTRODUCTION

Skeletal tissue loss caused by injury or disease such as trauma, tumour formation, limb preserving and reconstructive surgeries, and bone defects due to total joint replacement are a major health problem of our society. The necessity for procedures to generate cartilage and bone for clinical applications to heal skeletal defects is a big challenge in biology and medicine (Oreffo & Triffitt 1999; Bianco & Robey 2001). Current studies mainly rely on the use of fresh auto- and allografts or application of synthetic bone substitutes but these procedures have many limitations such as donor morbidity, limited tissue resources, lack of availability, or insufficient bioactivity (Marx & Morales 1988; Bonadio & Cunningham 2002). New strategies for skeletal tissue engineering are mainly focused on restoration of altered structures based on transplantation of cells in

Correspondence: Özer Degistirici, Center of Advanced European Studies and Research (Caesar), Ludwig-Erhard-Allee 2, D-53175 Bonn, Germany. Tel.: +49 228 9656 352; Fax: +49 228 9656 9352; E-mail: degistirici@caesar.de

combination with supportive matrix molecules (Awad *et al.* 2004; Nuttelman *et al.* 2004; Sittinger *et al.* 2004).

While use of human embryonic stem cells struggles with safety aspects concerning tumourigenicity and also raises serious ethical questions, adult stem cells and progenitors have received increasing scientific attention (Brower 1999; Erdo *et al.* 2003). Over the last decade, bone marrow-derived multipotent mesenchymal stem cells (MSCs) have been studied extensively for application in tissue engineering and regenerative medicine. MSCs have been shown to have limited proliferative capacity along with a clear capacity for mesodermal differentiation; however, with increasing age, numbers and differentiation capacity decrease. Furthermore, aspiration of bone marrow from a patient for isolation of MSCs is an invasive procedure (Caplan 1991; Pittenger *et al.* 1999; Rao & Mattson 2001; D'Ippolito *et al.* 2004; Mauney *et al.* 2005).

Human umbilical cord blood contains immature stem/progenitor cells that can be considered as an alternative allogenic source to bone marrow for stem cell tissue reconstitution (Gluckman *et al.* 1989; Broxmeyer *et al.* 1991, 1992; Rubinstein 1993). In addition, unrestricted somatic stem cells (USSCs) derived from cord blood provide new cell sources for pharmaceutical screening and have first encouraging results for cell therapy applications (Kogler *et al.* 2004, 2005). Besides other characteristics, USSCs can be stimulated to differentiate into cells of the three germ layer lineages, endodermal, ectodermal and mesodermal.

The goal of the study described here was to determine growth kinetics of USSCs in order to evaluate osteogenic and chondrogenic potential after isolation in early passages of USSCs, as well as after extensive subculture of these cells. One specific focus was to assess and quantify production of vascular endothelial growth factor (VEGF) and expression of VEGF receptors during osteogenic differentiation of USSCs.

MATERIALS AND METHODS

Isolation and culture of unrestricted somatic stem cells

Donated umbilical cord blood without criteria for non-commercial banking and with informed consent of the mothers, was used in this study (CB Bank, Düsseldorf, Germany) (Kogler *et al.* 2004, 2005), it was diluted 1 : 1 with phosphate-buffered saline (PBS, Cambrex Bio Science, Belgium). Mononuclear cells were separated from each cord blood sample by Ficoll density gradient centrifugation (density 1.077 g/cm³, Biochrom, Berlin, Germany) at 400 g for 25 min mononuclear cells in the interface were collected, washed twice with PBS centrifuged at 400 g for 10 min at 4 °C and were re-suspended with Dulbecco's modified Eagle's medium (DMEM)-low glucose (Cambrex Bio Science) supplemented with 30% foetal calf serum (FCS, selected charge, Biochrom) and 10⁻⁷ M dexamethasone as standard medium. Cells were plated at a density of 1 × 10⁶ cell/cm² and were cultured at 37 °C in a humidified chamber with 5% CO₂. After 24 h, half the culture medium was replaced with 50% fresh standard medium. Such medium changes were carried out every 7th day and culture conditions were evaluated every day by light microscopy. As soon as colony formation of fibroblastoid cells had reached a size of approximately 2 cm, these layers were detached from the flask's surface with 0.05% trypsin and were recultured/passaged using standard medium. At 80% confluence, adherent cells were passaged at 5000 cells/cm².

Bone marrow experiments were performed in accordance with rules of the local ethical committee and after informed consent of the donors; bone marrow was obtained from five healthy adults. The isolation protocol for mononuclear cells from bone marrow was in accordance with

the technique for cord blood cells described above (1×10^6 cells/cm² in DMEM-low glucose supplemented with 30% FCS and 10^{-7} M dexamethasone). Neonatal human dermal fibroblasts obtained from Cambrex Bio Science served as a control group.

Growth characteristics and population-doubling potential

Growth kinetics of cord blood-derived USSCs was measured at passage 2 and passage 6. Culture-expanded cells from the various cord blood samples were seeded in T25 flasks at a density of 2800 cells/cm², respectively. Duplicate cultures were harvested daily from each flask. Cell counts were performed and population doubling were calculated for each passage and sample (Bepler *et al.* 1987). Cell number and size were determined using an electronic cell counter (CASY; Shärfe-System, Reutlingen, Germany).

Flow cytometric analysis

Cells were harvested by trypsinization, washed twice with PBS containing 0.1% bovine serum albumin (BSA) and 0.01% sodium azide and were re-suspended in PBS. For staining, aliquots of $1\text{--}2 \times 10^5$ cells/100 μ L PBS–BSA were incubated with monoclonal antibodies tagged with fluorescent dye for 45 min. Thereafter, samples were washed twice with PBS–BSA and were analysed using the fluorescence activated cell sorter system, FACSARIATM (Becton Dickinson Biosciences, Heidelberg, Germany) by collecting 10 000 events. Flow cytometric data were analysed using FlowJo software (version 7, Tree Star Inc., Ashland, OR, USA). All antibodies used in this study are listed in Table 1. Isotypic antibodies (FITC, PE and APC, Becton Dickinson Bioscience) were used to define the extent of non-specific staining.

Differentiation potential of USSCs

Chondrogenic differentiation

For chondrogenic induction, we performed three-dimensional micromass cultures (pellet cultures) according to Johnstone *et al.* (1998). A total of 2×10^5 cells (USSCs, MSCs and fibroblasts) in standard growth medium was placed in a 15-mL polypropylene tube (BD Falcon, Heidelberg, Germany) and pelleted at 400 *g* for 5 min at 4 °C. Then, the medium was changed and samples were cultured in chondrogenic stimulation medium containing DMEM-high glucose (Cambrex Bio Science) supplemented with L-glutamine, 100 μ g/mL sodium-pyruvate, 50 μ g/mL L-ascorbic acid-2-phosphate, 10^{-7} M dexamethasone, 1% ITS (6.25 μ g/mL insulin, 6.25 μ g/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumine and 5.35 μ g/mL linoleic acid) + 1 and 10 ng/mL recombinant human transforming growth factor- β 1 (all from

Table 1. List of antibodies used for phenotyping of unrestricted somatic stem cells. Antibodies for flow cytometry application are used in conjunction with allophycocyanin (APC), phycoerythrin (PE) and fluorescein isothiocyanate (FITC) as fluorochrome

Antibody	Label	Company
CD13 (aminopeptidase)	APC	BD
CD14 (lipopolysaccharide receptor)	APC	BD
CD34	FITC	BD
CD44 (hyaluronate receptor)	FITC	BD
CD45 (leucocyte antigen)	PE	BD
CD49e (VLA- α 5)	PE	BD
Class II HLA (DR)	APC	BD

Sigma, St. Louis, MO, USA). Pellets were cultured at 37 °C in humidified air with 5% CO₂. The differentiation medium was changed every 2–3 days. Pellets were evaluated by biochemical, histological, immunohistochemical and reverse transcription-polymerase chain reaction (RT-PCR) for a period of 21 days.

Adipogenic differentiation

For adipogenic differentiation, USSCs were plated at a density of 8000 cells/cm². After initial expansion up to >80% confluence, as controlled by light microscope observation, cultures for adipogenic stimulation were treated with DMEM-low glucose (Cambrex Bio Science), 30% FCS (Biochrom), 10⁻⁶ M dexamethasone, 0.5 mM isobethylmethylenxanthin, 10 µg/mL insulin, 10 mM indomethacin (all from Sigma) as previously described (Pittenger *et al.* 1999).

Osteogenic differentiation

To promote osteogenic differentiation, cells (USSCs, MSCs and fibroblasts) were initially seeded at a density of 8000 cells/cm² and cultured in expansion medium. After reaching confluence of 70–80%, cultures were stimulated with differentiation medium, DMEM-low glucose (Cambrex Bio Science) supplemented with 30% FCS (Biochrom), 10⁻⁷ M dexamethasone, 50 µM ascorbic acid-2 phosphate and 10 mM β-glycerol phosphate (all from Sigma) according to Bruder *et al.* (1997). Medium was changed twice a week and cultures were analysed for a period of 28 days.

Biochemical analysis

Quantification of sulfated glycosaminoglycans

Sulfated glycosaminoglycans (sGAG) content was quantified after subculturing cells under chondrogenic conditions as described above. Pellet cultures were washed with PBS and then solubilized by digestion for 4 h at 65 °C with 25 µg/mL papain in 2 mM ethylenediaminetetraacetic acid, 50 mM sodium sulphate and 2 mM N-acetyl cystein (all from Sigma). For calculation of sGAGs, digests were stained with dimethylmethylene blue (Serva, Heidelberg, Germany) as previously described (Enobakhare *et al.* 1996). Samples were measured at 595 nm (Genios plate reader, TECAN, Crailsheim, Germany) in triplicate and content of sGAGs was expressed as µg sGAG/pellet.

Assay of alkaline phosphatase activity

Levels of alkaline phosphatase activity were determined in cell lysates using *p*-nitrophenyl phosphate as substrate (Sigma). The procedure was carried out as recommended by the manufacturer. Briefly, cells growing on 24-well plates (NUNC, Wiesbaden, Germany) were washed with PBS and incubated with 1% Triton X-100 (Sigma) for 30 min at 37 °C. The resulting lysate was then incubated with substrate and liberated *p*-nitrophenol (pNP) was measured at 405 nm (Genios plate reader, TECAN) and protein content of each lysate was measured using a commercial kit (Protein Assay; Bio-Rad, München, Germany). Alkaline phosphatase activity values were normalized to total protein. Alkaline phosphatase activity was expressed as nmol of pNP produced per minute per cell number or mg protein.

Quantification of calcium

Calcium measurements were performed as published previously (Jager *et al.* 2005). Cells growing on 24-well plates (NUNC) were washed with PBS and treated with 200 µL papain solution supplemented 50 mM sodium phosphate, 200 mM N-acetylcysteine, and 28 µg/mL papain (all from Sigma) for 1–16 h by 65 °C. Fifty microlitres aliquots from this lysate were analysed for

DNA content using a PicoGreen™ DNA quantification kit (Molecular Probes, Eugene, OR, USA). Total calcium of samples was measured by the o-cresolphthalein complexone method using the commercial Calcium Assay-Sigma Kit #587 (Sigma). The procedure was carried out according to the manufacturer's protocol. Briefly, aliquots from papain cell lysates (150 µL) were mixed with 150 µL of 1 N HCl (concentration 0.5 N). Then, samples were vigorously shaken for 4–16 h at 4 °C and centrifuged at 400 g for 5 min. The amount of deposited calcium was determined photometrically at 580 nm using a Genios plate reader (TECAN).

Histology and immunocytochemistry

Pellets generated by chondrogenic pellet culture assay were fixed with 4% paraformaldehyde for at least 30 min and processed blocks were cut into 5 µm sections. These were stained with 1% toluidine blue to determine presence of glycosaminoglycan and proteoglycan (Kavalkovich *et al.* 2002). For identification of extracellular matrix protein, the sections of pellet culture were incubated with 0.2 U/mL chondroitinase ABC (Sigma) and 1% BSA for 1 h. Sections were then incubated with mAb anticollagen type II at 1 : 200 dilution for 1 h at 37 °C and then rinsed with PBS. Specimens were further incubated with FITC-labelled secondary antibody (both antibodies from Chemicon, Souffelweyersheim, France) at 1 : 2500 for 1 h at 37 °C. Nuclei were stained with DAPI (Sigma). USSCs cultured in adipogenic medium were fixed with 4% paraformaldehyde for 30 min and were stained with Oil red O (Sigma) to identify lipid vacuoles, then were counterstained with haematoxylin. To identify mineralization, cultured cells were treated with alizarin red. Briefly, samples were rinsed in PBS, fixed with 70% ethanol (1 h, room temperature), washed with PBS and stained with fresh 1% alizarin red S (Sigma), pH 4.1 for 10 min at room temperature.

Isolation of RNA and RT-PCR

Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's specification. RNA was quantified spectrophotometrically using the Eppendorf Biophotometer (Eppendorf, Germany). Reverse transcription of RNA was carried out at 50 °C on total RNA of 100 ng using Omniscript RT Kits (Qiagen). Nucleotide sequences of all primers and probes are listed in Table 2. Expressions of various transcripts were assayed by PCR amplification using HotStar Taq Master Mix Kit (Qiagen) according to the manufacturer's instructions. Amplifications were performed for 35 cycles (typically: 94 °C/30 s, 55 °C/30 s, 72 °C/1 min) using Thermocycler (Biozym, Oldendorf, Germany). PCR products were separated by electrophoresis in 1% agarose gel (Roth, Karlsruhe, Germany) and the bands were identified by ultraviolet light.

***In vitro* vascular endothelial growth factor quantification**

Vascular endothelial growth factor was quantified using an antihuman VEGF ELISA (R&D Systems, Minneapolis, MN, USA). A total volume of 1 mL culture medium supernatants of osteogenic stimulated and non-stimulated controls were collected and assayed with quantitative sandwich ELISA according to manufacturer's instructions. All experiments were carried out in triplicate and are presented in relation to total DNA content of analysed cultures.

Statistics

Each assessment was performed on 2 or more independent cultures or pellets for each donor, cell source and experimental group. Mean values and standard deviation (SD) served as descriptive statistical parameters. To determine statistical significance, we performed the paired Student's *t*-test. *P*-values less than 0.05 were considered significant.

Table 2. List of primers used for RT-PCR, respectively

Primer name	Sequence	Accession number
β2-Microglobulin-5'	GTGGAGCATTGAGACTTGTC	BC032589
β2-Microglobulin-3'	AACAAGCTTTGAGTGCAAGAG	
AP2-5'	TGCCACCAGGAAAGTGGC	BC003672
AP2-3'	AACGTCCCTTGGCTTATGCTC	
PPARγ-5'	TCCATGCTGTTATGGGTGAAA	HSU79012
PPARγ-3'	TCACAAGCATGAACTCCATAG	
Osteocalcin-5'	CCTGGGGATGAGCTGGGGTGAAC	X04143
Osteocalcin-3'	CAAGGGCAAGGGGAAGAGGAAAGA	
PTHrP-R-5'	GGTCTGCCCCGCTGTCTTCGTG	HSU17418
PTHrP-R-3'	CATGGCAGGTGGTGTGGTCTCG	
Type X collagen-5'	TTTCTGGGACCCCTCTTGTTAGTGC	NM_000493
Type X collagen-3'	AGGGGGAAGGTTTGTGGTCTGATA	
Type IIA1 collagen-5'	AGACAGCATGACGCCGAGGTGGAT	HSC1A2CS
Type IIA1 collagen-3'	CGTGGACAGCAGGCGTAGGAAGGT	
COMP-5'	CTGGCTGTGGGTACACTG	NM_000095
COMP-3'	GATGATGTTCTCCTGGGAG	
BSP-5'	TGGGGTCTTTAAGTACAGGC	AB086984
BSP-3'	TTTGTATATCCCCAGCCTTC	
Chondroadherin-5'	AGGAACCAGCTGTCCAGCT	AF371328
Chondroadherin-3'	AGTCAACCAGGACTGGCTG	
PECAM-1-5'	CAGATAGTCGTATGTGAAATG	HUMPECAM1
PECAM-1-3'	CATTACAGCACATTGCAGC	
VEGFR2-5'	GGCAAAGACTACATTGTTC	AF063658
VEGFR2-3'	CTGCTTCCTCACTGGAGTAC	
VEGFR1-5'	GTCTTCCAGAAAGTGCATTG	AF063657
VEGFR1-3'	TGATAGATTTCAAGGAGTAGAG	

RESULTS

Isolation of USSCs

When kept under defined culture conditions in the presence of 30% FCS and 10^{-7} M dexamethasone, cord blood-derived mononuclear cells gave rise to a cell population with fibroblastic morphology (Fig. 1a). Colony formation (mean amount of 1.2 colonies/batch) was observed in 10 of the 34 processed cord blood samples and appeared approximately 10.4 days after initial plating (Fig. 1b,c). Colony formation did not correlate with gestation weeks, blood volume or numbers of mononuclear cells (Table 3). In contrast, mesenchymal stem cell colonies from bone marrow were found between days 3–5 in all five specimens with an average 19 colonies/ 10^6 mononuclear cells.

Characterization of USSCs

Growth kinetics of USSCs

In vitro growth characteristics of USSCs from passage 2 and from passage 6 are shown in Fig. 2. After an initial log phase of growth, cells proliferated with population doubling time of 39.5 h at passage 2 and of 43.4 h at passage 6 (Fig. 2a). To evaluate the maximum proliferation

Figure 1. Morphological features of cultured USSCs. (a) Appearance and growth of USSCs colony on day 8 (40x) and (b) passage 3 stem cells on day 3 (100x). (c) On day 7, cells showed fibroblast-like homogenous phenotype, respectively (100x).

capacity, two of the USSC batches were expanded to passage 16 and to passage 23 (Fig. 2b), thus representing 38 and 45 cell population doublings, respectively. With increase in passages, we observed an only moderate reduction in proliferative capacity and a change in morphology with a marked increase in cell size (Fig. 3a–e). At later passages, culture dishes showed local areas of osteogenically differentiated clusters.

Table 3. Cord blood conditions of cultures

	Isolation of USSCs	SD of mean	No. of USSCs colony	SD of mean
Week of gestation	39.5 (36–41)	1.8	39.7 (37–42)	1.27
Age of cord blood (CB) samples (h)	15.85 (4–25)	7.79	18.8 (8–31)	7.3
Number of mononuclear cells	1.08×10^8 ($3.2 \times 10^6 - 2 \times 10^8$)	1.05×10^8	1.6×10^8 ($3.8 \times 10^6 - 2.2 \times 10^8$)	2.1×10^8
Volume of CB (mL)	121.87 (71–263)	59.79	99.56 (58–223)	40.96
Number of colony	1.2 (1–2)	0.4	nd	nd
Appear of colony (days)	10.4 (7–12)	1.43	nd	nd
Total	10/34 (29.4%)		24/34 (70.6%)	

USSCs, unrestricted somatic stem cells; nd, none detected.

Figure 2. Growth curves of USSCs. (a) At day 0, 2800 cells/cm² from passage 2 and passage 6 were seeded in T25 flask. Cells were harvested every day for 7 days and subsequently counted. Results are expressed as mean from duplicate flasks of two different batches. (b) Expansion capacity of long-term cultures of USSCs. More than 20 passages (passage 23) without detectable differentiation could be obtained in cultures grow in medium containing high serum (30% FCS) with 10^{-7} M dexamethasone. Cells were trypsinized at 80%–90% confluence and seed further with 5000 cells/cm².

Cell surface analysis of USSCs

USSCs were analysed for expression of various surface antigens, as indicated in Fig. 4. USSCs stained positively for CD13, CD44 and CD49e. In addition, the cells were negative for CD14, CD34, CD45 and class II human leukocyte antigen (HLA).

Figure 3. Cell morphology and cell size of long term cultivation. (a) Cell phenotypes were observed in passage 4, (b) passage 10, (c) passage 15, and (d) passage 23 (all 100x). (e) The cell size from different passages (n: 3).

Differentiation potential of USSCs

Chondrogenic differentiation of USSCs

The chondrogenic differentiation potential of USSCs and bone marrow-derived MSCs was analysed for cells from passage 2 or 3. USSCs formed spherical bodies in pellet culture system within 24 h (Johnstone *et al.* 1998) (Fig. 5a). Microsectioning of pellets showed proteoglycan production as documented by toluidine blue staining at 21 days after stimulation (Fig. 5b). In addition, immunohistochemical staining of type II collagen at 21 days after stimulation revealed expression of this extracellular matrix protein (Fig. 5c).

The status of USSC differentiation towards the chondrogenic lineage was further monitored by analysing the transcription pattern of chondro-specific genes by RT-PCR. Here, the presence

Figure 4. USSCs were labeled with directly labeled (FITC, PE, APC) antibodies against different cell surface markers. Red line, control IgG; green line specific antibody.

of four extracellular matrix protein mRNAs [type II collagen, type X collagen, cartilage oligo-matrix protein (COMP) and chondroadherin] was determined. At short-time intervals (days 0, 4, 7, 9, 11, 14, 16, 18, 21); COMP could be detected on day 0 and showed a steady increase during chondrogenic stimulation. Chondroadherin and type II collagen were detected first at day 7 and increased expression with prolonged stimulation. Type X collagen was detected strongly at day 0 and steadily decreased following chondrogenic cultivation (Fig. 5d).

Synthesis of sGAG was measured as a marker of chondrogenic differentiation. USSCs were compared to bone marrow-derived MSCs and fibroblasts during chondrogenic differentiation. sGAG content of samples was assayed using the dimethylmethylene blue colorimetric assay at 7, 14 and 21 days. USSCs and MSCs exhibited a steady increase in sGAG content over the

Figure 5. USSCs induced toward the chondrogenic lineage in pellet culture synthesized a cartilagenous matrix and expressed gene consistent with their chondrogenic lineage differentiation. (a) USSCs were induced in chondrogenic medium under micromass culture conditions after one day. (b) Pellets after 21 days were sectioned and stained methachromatic with toluidine blue (40x) and (c) immunostained with antibody directed against type II collagen (200x), Nuclei are marked with DAPI (blue). (d) RT-PCR analysis of cartilage oligomatrix protein (COMP), chondroadherin, type II collagen, and type X collagen in the cells incubated in chondrogenic medium during three weeks. β -2-microglobulin (β 2M) served as a housekeeping gene. (e) Time course of USSCs, BMSCs, and fibroblasts chondrogenic cultures seeded at 2×10^5 cells/pellets. Accumulation of sulfated-glycosaminoglycan (sGAG) in pellet cultured in chondrogenic medium over 21 days. The total amount of extracted DMMB-reactive glycosaminoglycan per pellet was determined. Asterisks denote a significant difference (USSCs, $n = 8$; BMSCs, $n = 5$), ** $P < 0.05$, * $P < 0.001$, samples were compared with day 0 start cultures.

course of the experiment with significant differences between early and the later time points, while fibroblasts had almost no sGAG production after chondrogenic stimulation (Fig. 5e).

Adipogenic differentiation of USSCs

For adipogenic differentiation, USSCs were cultured in adipogenic medium for 14 days; adipogenic differentiation was identified by Oil red O staining. All six USSCs batches, when stimulated towards adipogenic lineage exhibited many typical neutral lipid vacuoles within their cytoplasm,

Figure 6. The USSCs cultured for 14 days in adipogenic medium and in control medium. (a) The presence of lipid vesicles accumulated in the cytoplasm of adipocytes was detected by staining for Oil red O (200x) (b) while control cultures did not show any staining (200x). (c) RT-PCR analysis of gene expression of peroxisome proliferator-activated receptor gamma (PPAR γ) and fatty acid binding protein 2 (aP2). USSCs induced towards the adipogenic lineage expressed the PPAR γ weakly at day 7 and aP2 weakly at day 7 and highly at day 14. β -2-microglobulin (β 2M) served as a housekeeping gene.

as seen for mature adipocytes (Fig. 6a). Cells maintained with expansion medium did not stain with Oil red O (Fig. 6b). RT-PCR further revealed expression of adipocyte-specific genes, the transcription factor peroxisome proliferator-activated receptor- γ (PPAR γ) and fatty acid binding protein (aP2), after adipogenic stimulation. PPAR γ was weakly detectable at day 7, while aP2 expression was found at days 7 and 14 (Fig. 6c).

Osteogenic differentiation of USSCs

To evaluate osteogenic differentiation capacity, USSCs were plated and treated for osteogenic differentiation as described for up to 21 days. Osteogenic differentiation was evaluated using morphological, biochemical and molecular biological techniques. Under osteogenic stimulation, hydroxyapatite-associated calcium mineral accumulation in the extracellular matrix of USSCs became evident at day 14 of culture (Fig. 7a) and steadily increased for up to 21 days (Fig. 7b). Extracellular matrix was stained with alizarin red, demonstrating amorphous deposits observed by microscopy were calcium deposits. Unstimulated controls did not show any calcium accumulation (Fig. 7c).

Proliferative behaviour of USSCs during osteogenic differentiation revealed that the cells caused proliferative activity after osteogenic stimulation while control cultures continued to proliferate (Fig. 7d). During osteogenic stimulation, USSCs and MSCs both showed formation of calcium deposits significantly above values for unstimulated and osteogenically stimulated fibroblasts (Fig. 7e). Activity of alkaline phosphatase was elevated significantly in osteogenic stimulation at 4 days and 7 days in comparison to the control cultures of the same days, then decreased to the value of those control cultures (Fig. 7f).

Figure 7. Osteogenic differentiation of USSCs. (a) Alizarin red staining of hydroxyapatite-associated calcium mineral deposited in the extra cellular matrix after 14 days, (b) and after 21 days. (c) The unstimulated cultures did not stain with alizarin red. Magnifications are 40x for (a) and (b), and 200x for (c). (d) Proliferative behaviour of USSCs during osteogenic differentiation. DNA content of cultivated cells for determination of proliferation was quantified with PicoGreen from independent cultures (n: 4). (e) The calcium content of USSCs, BMSCs and fibroblast-cultures were determined with quantitative calcium assay and normalized to the DNA content. Asterisk denotes (n: 6 for USSCs, n: 3 for BMSCs) a significant difference, $*P < 0.01$, compared from same day of control cultures. (f) ALP activity of osteogenic stimulated USSCs cultures and control cultures were determined and normalized with respect to cell number. Asterisk denotes (n: 3) a significant difference, $**P < 0.05$, compared from same day of control cultures.

Analysis of long-term expanded USSCs for osteogenic and chondrogenic potential

We evaluated one batch of USSCs beyond 21 passages for its differentiation towards osteogenic and chondrogenic lineages. We observed that they responded positively to osteogenic and chondrogenic stimulation. However, the quantitative calcium assay showed that osteogenic potential decreased over time by 40% for passage 21 compared to passage 6, while alkaline phosphatase activity of controls remained steady, almost unchanged and stimulated cultures showed strong alkaline phosphatase activity on day 7, however failed lower than in early passages (Fig. 8a,b).

Figure 8. *In vitro* differentiation of long-term cultures of USSCs. (a,b) Alkaline phosphatase (ALP) activity and calcium content of cell-matrix layers were determined during cultivation of passage 6, passage 15, and passage 21. (c) sGAGs were quantified per pellet from passage 6, passage 15, and passage 21 after chondrogenic stimulation.

The chondrogenic differentiation potential of USSCs showed a decrease in sGAG content by 30% for passage 21 compared to passage 6 stem cells (Fig. 8c).

Analysis of VEGF production and gene expression of osteogenic and angiogenic cells during osteogenic differentiation of USSCs

Vascular endothelial growth factor is known to be an angiogenic factor. Production and secretion of VEGF protein in culture medium was measured by ELISA. Levels of VEGF increased highly after osteogenic stimulation for 14 days compared to start culture and control culture on day 14. VEGF protein could be detected in control cultures but less when compared to osteogenic stimulated cultures (Fig. 9a).

Reverse transcription-polymerase chain reaction further revealed expression of specific osteogenic differentiation marker genes, such as bone sialoprotein (BSP), osteocalcin and parathyroid hormone-related protein receptor (PTHrP-R), during osteogenic stimulation. Expression rates of osteocalcin were scarcely detectable at day 0, but clearly detectable by day 4, day 7 and day 14. BSP and PTHrP-R were detectable first on day 4 of induction and increased further. To confirm the angiogenic potential of USSCs after osteogenic stimulation, cells were examined for angiogenic markers such as vascular endothelial growth factor receptor-1 (VEGFR1), the vascular endothelial growth factor receptor-2 (VEGFR2) and platelet/endothelial cell adhesion molecule (PECAM; CD31). USSCs showed low levels of VEGFR1 expression in osteogenic stimulated cultures as well as in control cultures. Expression of VEGFR2 was detected at low levels on day 4 and by a great extent at day 14 after osteogenic stimulation. PECAM, a marker of endothelial differentiation, was detected only at 14 days, while unstimulated cultures did not show any expression of it (Fig. 9b).

DISCUSSION

There is increasing interest in tissue-engineering concepts and cell therapy approaches to restore bone and cartilage functionality. Current strategies mainly consist of autologous cell therapeutics for tissue-specific somatic cells with or without a biomaterial. However, stem cell-based therapies are becoming a reliable alternative. Here, bone marrow-derived MSCs, the most investigated adult stem cell population (Pittenger *et al.* 1999; Quarto *et al.* 2001), have been compared to USSCs. We have evaluated isolation and mesodermal differentiation processes of these unrestricted somatic stem cells. They can be isolated from cord blood and prove to have excellent proliferative capacity characteristics together with broad differentiation potential.

In comparison to bone marrow-derived MSCs (10^6 mononuclear cells/19 colonies), success rates for generation of USSCs were very limited. Only 29% of all cord blood batches exhibited colony formation, with 1–2 colonies being generated per batch. These colonies appeared later than colonies from bone marrow, thus indicating an early developmental stage of this cell type. USSCs showed enormous proliferative capacity (here passage 23) next to strong differentiation capacity into osteogenic and chondrogenic lineages, even at high passages. However, we observed that cell size increased significantly after long-term proliferation and osteogenic nodule-like formation appeared spontaneously at the end of proliferation activity (Peterson *et al.* 2004; Vacanti *et al.* 2005). Thus, unlike embryonic cells, USSCs cannot grow indefinitely (Hoffman & Carpenter 2005).

Induction of chondrogenesis in USSCs was carried out in pellet cultures as reported previously for MSCs from bone marrow (Johnstone *et al.* 1998; Mackay *et al.* 1998). Development towards

Figure 9. Vascular endothelial growth factor (VEGF) protein production of USSCs during osteogenic differentiation and RT-PCR analysis. (a) Protein content was assayed in culture supernatants by VEGF-ELISA at the time point indicated and normalized to DNA content (n: 4). (b) RT-PCR analysis of the expression of genes related to the osteogenic and vasculogenic lineage differentiation after osteogenic stimulation. USSCs express the osteoblastic phenotype markers osteocalcin, bone sialoprotein (BSP) and parathyroid hormone-related protein receptor (PTHrP-R). Moreover, control cells and osteogenic stimulated USSCs expressed vascular endothelial growth factor receptor 1 (VEGFR1), while osteogenic stimulated USSCs expressed vascular endothelial growth factor receptor 2 (VEGFR2) and platelet/endothelial cellular adhesion molecule (PECAM) on day 14. β -2-microglobulin (β 2M) served as a housekeeping gene.

the chondroblast phenotype was investigated by immunohistochemical staining of micromass sections for type II collagen and by histological staining with toluidine blue. Quantitative sGAG production after chondrogenic stimulation of USSCs was comparable to that of MSCs from bone marrow and was significantly higher than that of fibroblasts (Barry *et al.* 2001; Kavalkovich *et al.* 2002). RT-PCR analysis indicated that specific chondrogenic marker genes for type II collagen and chondroadherin were up-regulated from day 7. Type X collagen, a key marker for hypertrophic cartilage (Erlebacher *et al.* 1995) whose expression has been reported in endochondral ossification by bone marrow stromal cells (Jacenko *et al.* 1996), was investigated. In contrast to MSCs from bone marrow and from fatty tissue, we detected high levels of type X collagen at day 0 and subsequent down regulation during differentiation (Johnstone *et al.* 1998; Barry *et al.* 2001; Betre *et al.* 2006). Weak expression of type X collagen as well as strong expression of type II collagen and chondroadherin, point out that in culture still no hypertrophic chondrocytes were detectable. We thus hypothesized that USSCs can be an alternative source for regeneration of cartilage tissue and no hypertrophic chondrocytes were built.

In common with other groups, we found adipogenic differentiation of USSCs as indicated by formation of lipid vesicles that very strongly stained with Oil red O. Furthermore, characteristic marker genes of adipogenic differentiation such as that for PPAR γ (detected weakly at day 7) and aP2 (detected at day 14) detail developmental stages during adipogenic differentiation (Pittenger *et al.* 1999; Jaiswal *et al.* 2000).

Dexamethasone is a synthetic glycocorticoid that increases osteogenic differentiation on bone marrow-derived MSCs (Jaiswal *et al.* 1997; Ter Brugge & Jansen 2002). Although dexamethasone is a potent induction agent, USSCs can proliferate in dexamethasone supplemented medium. When in culture medium in the presence of ascorbic acid-2-phosphate and β -glycerolphosphate, cells start to differentiate rapidly into bone cells and stop proliferating. Several previous studies have reported that culture of MSCs in osteogenic medium (dexamethasone, ascorbic acid-2-phosphate and β -glycerolphosphate) induce large amounts of mineralized matrix to be formed and have demonstrated high activity of alkaline phosphatase at day 7 and day 10 (Jaiswal *et al.* 1997, 2000; Pittenger *et al.* 1999; Zuk *et al.* 2002). We have observed recently similar results for USSCs. In contrast to building nodules during osteogenic differentiation like MSCs, USSCs had speared calcium accumulation and showed uniformity of calcium accumulation on the surface layer of cells at 21 days of differentiation, similar to development of calvaria and long-bone diaphyses (Bruder & Caplan 1989). Commitment of USSCs towards osteogenic differentiation was demonstrated by expression of osteocalcin, a protein involved in deposition of new extracellular matrix and essential for calcium binding. In addition, BSP, an adhesive bone extracellular protein, associated with mature osteoblasts and involved in the mineralization phase of bone formation and PTHrP-R (parathyroid hormone-related receptor), indicating osteogenic differentiation up to day 4 after osteogenic stimulation of USSCs.

Angiogenesis is a key process of fracture healing. VEGF and VEGF receptors are also important components in regulation of angiogenesis during fracture healing (Gerber *et al.* 1999; Street *et al.* 2002; Gerstenfeld *et al.* 2003). Production of VEGF and expression of VEGFR1 and VEGFR2 during osteogenic differentiation of MSCs from bone marrow have been reported in a variety of studies (Deckers *et al.* 2000; Fiedler *et al.* 2005; Maes *et al.* 2006), yet some studies have shown no expression of VEGFR1, VEGFR2 during osteogenic differentiation of MSCs (Furumatsu *et al.* 2003). For USSCs, secretion of VEGF could be detected in control cultures and to a greater extent in osteogenically stimulated cultures. VEGFR1 could be detected, in comparison to human MSCs in control cultures and in stimulated cultures, while VEGFR2 was detected only in osteogenic stimulated cultures. Unexpectedly, we found high expression of PECAM (CD31), which otherwise is mainly restricted to endothelial cells (Albelda *et al.* 1991;

Newman 1994). Thus, expression and secretion of VEGF and expression of VEGFR1, VEGFR2 and PECAM suggest that USSCs may play an active role in vascularization and might be an ideal source in bone repair strategies.

In conclusion, we are able to document strong capacity of cord blood-derived USSCs towards osteogenic lineage accompanied by induction of angiogenesis and vasculogenesis, which makes this source of stem cells ideal for cell therapeutics for bone repair strategies. In addition, we showed stable differentiation towards the chondrogenic lineage. Because both differentiation pathways were shown to be stable, even at higher passages, this stem cell source indicates its applicability for a high-throughput manufacturing process in an allogenic setting.

ACKNOWLEDGEMENTS

The skilful technical assistance of Inge Napierski and Stephan Schroot is acknowledged with great gratitude. We also would like to thank PD Dr. Stephan Wnendt/CEO of Kourion Therapeutics for supporting this work. The authors thank Prof. Dr. Ivan Martin (University Basel, Switzerland) for a critical reading of the manuscript. Sincere thanks also to Prof. Dr. Peter Wernet and Prof. Dr. Gesine Kögler for their encouragement at the beginning of this work.

REFERENCES

- Albelda SM, Muller WA, Buck CA, Newman PJ (1991) Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. *J. Cell Biol.* **114**, 1059–1068.
- Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F (2004) Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* **25**, 3211–3222.
- Barry F, Boynton RE, Liu B, Murphy JM (2001) Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. *Exp. Cell Res.* **268**, 189–200.
- Bepler G, Jaques G, Neumann K, Aumuller G, Gropp C, Havemann K (1987) Establishment, growth properties, and morphological characteristics of permanent human small cell lung cancer cell lines. *J. Cancer Res. Clin. Oncol.* **113**, 31–40.
- Betre H, Ong SR, Guilak F, Chilkoti A, Fermor B, Setton LA (2006) Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide. *Biomaterials* **27**, 91–99.
- Bianco P, Robey PG (2001) Stem cells in tissue engineering. *Nature* **414**, 118–121.
- Bonadio J, Cunningham ML (2002) Genetic approaches to craniofacial tissue repair. *Ann. N. Y. Acad. Sci.* **961**, 48–57.
- Brower V (1999) Human ES cells: can you build a business around them? *Nat. Biotechnol.* **17**, 139–142.
- Broxmeyer HE, Kurtzberg J, Gluckman E, Auerbach AD, Douglas G, Cooper S, Falkenburg JH, Bard J, Boyse EA (1991) Umbilical cord blood hematopoietic stem and repopulating cells in human clinical transplantation. *Blood Cells* **17**, 313–329.
- Broxmeyer HE, Hangoc G, Cooper S (1992) Clinical and biological aspects of human umbilical cord blood as a source of transplantable hematopoietic stem and progenitor cells. *Bone Marrow Transplant.* **9**(Suppl. 1), 7–10.
- Bruder SP, Caplan AI (1989) Cellular and molecular events during embryonic bone development. *Connect. Tissue Res.* **20**, 65–71.
- Bruder SP, Jaiswal N, Haynesworth SE (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J. Cell. Biochem.* **64**, 278–294.
- Caplan AI (1991) Mesenchymal stem cells. *J. Orthop. Res.* **9**, 641–650.
- D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J. Cell Sci.* **117**, 2971–2981.

- Deckers MM, Karperien M, van der BC, Yamashita T, Papapoulos SE, Lowik CW (2000) Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. *Endocrinology* **141**, 1667–1674.
- Enobakhare BO, Bader DL, Lee DA (1996) Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal. Biochem.* **243**, 189–191.
- Erdo F, Buhrle C, Blunk J, Hoehn M, Xia Y, Fleischmann B, Focking M, Kustermann E, Kolossov E, Hescheler J, Hossmann KA, Trapp T (2003) Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. *J. Cereb. Blood Flow Metab.* **23**, 780–785.
- Erlebacher A, Filvaroff EH, Gitelman SE, Derynck R (1995) Toward a molecular understanding of skeletal development. *Cell* **80**, 371–378.
- Fiedler J, Leucht F, Waltenberger J, Dehio C, Brenner RE (2005) VEGF-A and PlGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. *Biochem. Biophys. Res. Commun.* **334**, 561–568.
- Furumatsu T, Shen ZN, Kawai A, Nishida K, Manabe H, Ohashi T, Inoue H, Ninomiya Y (2003) Vascular endothelial growth factor principally acts as the main angiogenic factor in the early stage of human osteoblastogenesis. *J. Biochem. (Tokyo)* **133**, 633–639.
- Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat. Med.* **5**, 623–628.
- Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA (2003) Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J. Cell. Biochem.* **88**, 873–884.
- Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, Esperou H, Thierry D, Socie G, Lehn P (1989) Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. *N. Engl. J. Med.* **321**, 1174–1178.
- Hoffman LM, Carpenter MK (2005) Characterization and culture of human embryonic stem cells. *Nat. Biotechnol.* **23**, 699–708.
- Jacenko O, Ito S, Olsen BR (1996) Skeletal and hematopoietic defects in mice transgenic for collagen X. *Ann. N. Y. Acad. Sci.* **785**, 278–280.
- Jager M, Feser T, Denck H, Krauspe R (2005) Proliferation and osteogenic differentiation of mesenchymal stem cells cultured onto three different polymers *in vitro*. *Ann. Biomed. Eng.* **33**, 1319–1332.
- Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J. Cell. Biochem.* **64**, 295–312.
- Jaiswal RK, Jaiswal N, Bruder SP, Mbalaviele G, Marshak DR, Pittenger MF (2000) Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J. Biol. Chem.* **275**, 9645–9652.
- Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* **238**, 265–272.
- Kavalkovich KW, Boynton RE, Murphy JM, Barry F (2002) Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system. *In Vitro Cell. Dev. Biol. Anim.* **38**, 457–466.
- Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Meida-Porada G, Muller HW, Zanjani E, Wernet P (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J. Exp. Med.* **200**, 123–135.
- Kogler G, Radke TF, Lefort A, Sensken S, Fischer J, Sorg RV, Wernet P (2005) Cytokine production and hematopoiesis supporting activity of cord blood-derived unrestricted somatic stem cells. *Exp. Hematol.* **33**, 573–583.
- Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF (1998) Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.* **4**, 415–428.
- Maes C, Coenegrachts L, Stockmans I, Daci E, Luttun A, Petryk A, Gopalakrishnan R, Moermans K, Smets N, Verfaillie CM, Carmeliet P, Bouillon R, Carmeliet G (2006) Placental growth factor mediates mesenchymal cell development, cartilage turnover, and bone remodeling during fracture repair. *J. Clin. Invest.* **116**, 1230–1242.
- Marx RE, Morales MJ (1988) Morbidity from bone harvest in major jaw reconstruction: a randomized trial comparing the lateral anterior and posterior approaches to the ilium. *J. Oral Maxillofac. Surg.* **46**, 196–203.
- Mauney JR, Jaquiere C, Volloch V, Heberer M, Martin I, Kaplan DL (2005) *In vitro* and *in vivo* evaluation of differentially demineralized cancellous bone scaffolds combined with human bone marrow stromal cells for tissue engineering. *Biomaterials* **26**, 3173–3185.
- Newman PJ (1994) The role of PECAM-1 in vascular cell biology. *Ann. N. Y. Acad. Sci.* **714**, 165–174.
- Nuttelman CR, Tripodi MC, Anseth KS (2004) *In vitro* osteogenic differentiation of human mesenchymal stem cells photoencapsulated in PEG hydrogels. *J. Biomed. Mater. Res. A* **68**, 773–782.
- Oreffo RO, Triffitt JT (1999) Future potentials for using osteogenic stem cells and biomaterials in orthopedics. *Bone* **25**, S5–S9.

- Peterson WJ, Tachiki KH, Yamaguchi DT (2004) Serial passage of MC3T3-E1 cells down-regulates proliferation during osteogenesis *in vitro*. *Cell Prolif.* **37**, 325–336.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147.
- Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, Kon E, Marcacci M (2001) Repair of large bone defects with the use of autologous bone marrow stromal cells. *N. Engl. J. Med.* **344**, 385–386.
- Rao MS, Mattson MP (2001) Stem cells and aging: expanding the possibilities. *Mech. Ageing Dev.* **122**, 713–734.
- Rubinstein P (1993) Placental blood-derived hematopoietic stem cells for unrelated bone marrow reconstitution. *J. Hematother.* **2**, 207–210.
- Sittinger M, Hutmacher DW, Risbud MV (2004) Current strategies for cell delivery in cartilage and bone regeneration. *Curr. Opin. Biotechnol.* **15**, 411–418.
- Street J, Bao M, deGuzman L, Bunting S, Peale FV Jr, Ferrara N, Steinmetz H, Hoeffel J, Cleland JL, Daugherty A, van BN, Redmond HP, Carano RA, Filvaroff EH (2002) Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc. Natl. Acad. Sci. USA* **99**, 9656–9661.
- Ter Brugge PJ, Jansen JA (2002) *In vitro* osteogenic differentiation of rat bone marrow cells subcultured with and without dexamethasone. *Tissue Eng.* **8**, 321–331.
- Vacanti V, Kong E, Suzuki G, Sato K, Canty JM, Lee T (2005) Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. *J. Cell. Physiol.* **205**, 194–201.
- Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* **13**, 4279–4295.